

CLAIMS

1. Method for evolving an X protein encoded by a *Lactobacillus fermentum* (*L. fermentum*) *ntd* gene so as to modify its characteristics,
 5 comprising the following stages:
 - a) obtaining mutants of the *L. fermentum ntd* gene by random mutagenesis;
 - b) transformation of cells comprising a [P-] phenotype with vectors
 comprising the mutated nucleic acid obtained in stage a) coding for the
 thus modified X* proteins, P- meaning that said cells are auxotrophic for
 10 the substance P, P being the product of the action of X on its natural
 substrate S;
 - c) culture of said cells in a medium comprising a substrate S*,
 S* being an analogue of the natural substrate S of said X protein;
 - d) selection of the cells [P-:: X*] which have survived stage c) in which the
 15 X* proteins are capable of carrying out the biosynthesis of the product P
 from the substrate S*.
2. Method according to claim 1, characterized in that the mutant X*
 protein obtained is a protein possessing an activity similar to said protein X, i.e.
 belonging to the same or adjacent enzyme classes having at least the first
 20 three figures 2.4.2 of the EC 4-figure international nomenclature classes.
3. Method according to one of claims 1 and 2, characterized in that the
 cells used in stage b) are obtained by the inactivation of at least one gene
 involved in the natural metabolic pathway leading to the product P.
4. Method according to claim 3, characterized in that the protein X*
 25 complements the deficiency of the natural metabolic pathway leading to the
 product P in a medium provided with the substrate S*.
5. Method according to one of claims 1 to 4, characterized in that the
 activity of the protein X on the substrate S is at least two times greater than its
 activity on the substrate S*.
- 30 6. Method according to one of claims 1 to 5, characterized in that the
 activity of the protein X* on the substrate S* is at least 10 times greater than
 its activity on the substrate S.
7. Method according to one of claims 1 to 6, characterized in that the
 random mutagenesis of stage a) is carried out either by variation of the

manganese concentration during the PCR reaction, or by the use of promutagenic nucleotide analogues or also by the utilization of primers comprising a random sequence.

8. Method according to one of claims 1 to 7, characterized in that said
5 cells are prokaryotic or eukaryotic cells, preferably *E. coli*.

9. Method according to one of claims 1 to 8 characterized in that an N-deoxyribosyl transferase (DTP) of *L. fermentum* is evolved so as to obtain a protein which is an N-dideoxyribosyl transferase by the following stages:

- a) obtaining DTP* mutants of the sequence coding for an N-deoxyribosyl
10 transferase (DTP) by random mutagenesis;
- b) transformation of cells comprising an [N-] phenotype with vectors comprising the mutated nucleic acid obtained in stage a) coding for the DTP* proteins, N- meaning that said cells are auxotrophic for at least one nucleoside, said nucleoside being the product of the action of DTP
15 on its natural substrate dR-N;
- c) culture of said cells in a medium comprising a ddR-N substrate;
- d) selection of the [N-:: DTP*] cells which have survived stage c) in which the DTP* proteins are capable of carrying out the transfer of the dideoxyribose (ddR) from a dideoxyribonucleoside to another nucleoside
20 leading to the production of the N nucleoside necessary for the survival of the cells.

10. Method according to claim 9 characterized in that the (*ntd*) sequence encoding the N-deoxyribosyl transferase (DTP) of *L. fermentum* corresponds to SEQ ID No. 1 which is being evolved.

25 11. Method according to one of the claims 9 and 10 characterized in that the cells used in stage b) are bacteria of genotype $\Delta pyrC$, $\Delta codA$, Δcdd deficient in the metabolic pathway leading to uracil.

12. Method according to claim 11, characterized in that the bacteria of genotype $\Delta pyrC$, $\Delta codA$, Δcdd deficient in the metabolic pathway leading to
30 uracil used are *E. coli*.

13. Mutated N-deoxyribosyl transferase protein (DTP) capable of being obtained from the method according to one of claims 1 to 12, characterized in that it has a modified activity.

14. Protein according to claim 13, characterized in that it has an N-dideoxyribosyl transferase activity and/or an activity on deoxy or dideoxyribonucleoside analogues comprising a modified base.

5 15. Protein according to claim 13 or 14, characterized in that it has a sequence at least 70% identical to SEQ ID No. 2 and containing the residues Y13, D77, D97, E103, M132.

16. Protein according to claim 15, characterized in that it has a sequence identity with SEQ ID No. 2 greater than or equal to 80%.

10 17. Protein having an N-dideoxyribosyl transferase activity according to any one of claims 14 to 16, characterized in that its sequence comprises SEQ ID No. 4.

15 18. Protein having an activity on deoxy- or dideoxyribonucleoside analogues, having a percentage identity with SEQ ID No. 4 equal to or greater than 70%, and comprising a threonine residue corresponding to the mutation point A15T of SEQ ID No. 4.

19. Protein according to claim 18, characterized in that it has a percentage identity with SEQ ID No. 4 equal to or greater than 80%.

20 20. Protein according to one of claims 18 and 19, characterized in that the sequence of said protein moreover comprises the residues corresponding to Y13, D77, D97, E103 and M132 of SEQ ID No. 4.

21. Protein according to any one of claims 18 to 20, characterized in that said protein has an N-dideoxyribosyl transferase activity.

25 22. Protein according to any one of claims 18 to 21, characterized in that said protein has a deoxyribose and dideoxyribose and/or didehydroribose transfer activity .

23. Protein according to any one of claims 18 to 22, characterized in that said protein has a catalytic activity on d4T and ddT greater than that of the native N-deoxyribosyl transferase protein of *L. fermentum* represented by SEQ ID No. 2.

30 24. Protein according to claim 23, characterized in that said catalytic activity on d4T and ddT is 50% greater than that of the native N-deoxyribosyl transferase protein of *L. fermentum* represented by SEQ ID No. 2.

25. Protein according to any one of claims 18 to 24, characterized in that said protein has a catalytic effectiveness on d4T and ddT greater than that of

the native N-deoxyribosyl transferase protein of *L. fermentum* represented by SEQ ID No. 2.

26. Protein according to claim 25, characterized in that said catalytic effectiveness on d4T and ddT is at least 5 times greater than that of the native
5 N-deoxyribosyl transferase protein of *L. fermentum* represented by SEQ ID No. 2.

27. Protein according to any one of claims 19 to 26, characterized in that it consists of a polypeptide of sequence SEQ ID No. 4.

28. Nucleic acid comprising a sequence coding for a protein having an
10 N-dideoxyribosyl transferase activity according to any one of claims 13 to 27, such as the sequence SEQ ID No.3.

29. Expression vector comprising a nucleic acid according to claim 28.

30. Vector according to claim 29, characterized in that the nucleic acid of claim 28 is fused to an effective promoter for the expression of said coding
15 sequence in the eukaryotic and/or prokaryotic cells.

31. Vector according to one of claims 29 and 30, characterized in that it is a plasmid capable of transforming and being maintained in *E. coli*.

32. Host cell comprising a vector according to one of claims 29 to 31.

33. Use of a protein having an N-dideoxyribosyl transferase activity
20 according to any one of claims 13 to 27 for the transfer of a dideoxyribose (ddR) from a dideoxyribonucleoside to another nucleoside.

34. Use according to claim 33, in the synthesis of 2',3'-dideoxynucleosides.

35. Use according to claim 33, in the synthesis of 2',3'-didehydro-2',3'-
25 dideoxynucleosides.

36. Use according to any one of claims 32 to 35 for the preparation of nucleoside or nucleotide analogues possessing anti-tumor properties.

37. Use according to claim 36 for the preparation of ddl or ddC.

38. Method for the preparation of compounds comprising a stage
30 consisting of utilizing a mutated protein according to one of claims 13 to 27.

39. Method according to claim 38 for the preparation of nucleoside or nucleotide analogues useful for the treatment of cancer or infectious diseases, such as dideoxyribonucleosides, such as ddC and ddl or didehydro-dideoxyribonucleosides.

40. Strain of *E. coli* deposited at the CNCM on 22nd March 2004 under accession number I-3192.